COMPARISON OF THREE METHODS TO ANALYZE NON-AROMATIC ORGANIC ACIDS IN HONEY

Mato I[1]; Huidobro JF[1]; Simal-Lozano J[1];

Fernández-Muiño MA[2] & Sancho MT[2]

[1]University of Santiago de Compostela (Spain)

[2]University of Burgos (Spain)

IMPORTANCE OF THE DETERMINATION OF ORGANIC ACIDS IN HONEY *(Mato et al. 2003. J. Food Prot. 66)*

Organic acids ⟶ Minor constituents of honey

Important contributions to honey properties

Antibacterial activity ⟶ **Free and total acidity**

(Bogdanov. 1997. Lebensm. Wiss. Technol. 30)

Antioxidant activity ⟶ **Organic acids**

(Gheldof et al. 2002. J. Agric. Food Chem. 50)

Indicators of fermentation ⟶ **Acetic acid**

(Gonnet. 1982. Opida INRA 2nd ed.)

Treatment against *Varroa* infestation ⟶
- **Formic acid**
- **Lactic acid**
- **Oxalic acid**

(Gregorc and Planinc. 2001. Apidologie 32)

Factors for the characterization of botanical and geographical origins

(Anklam. 1998. Food Chem. 63)

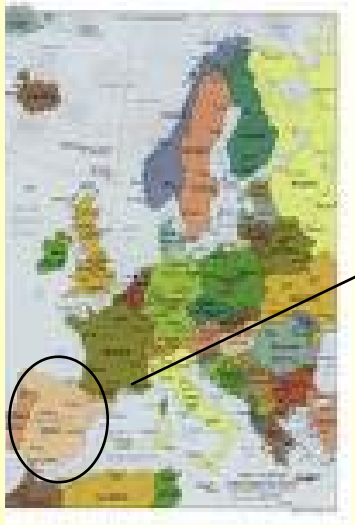
PURPOSE:

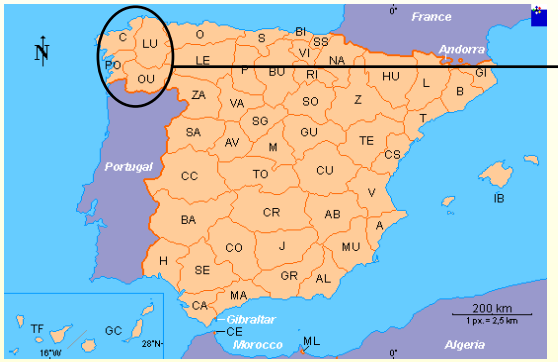
To compare **enzymatic**, **HPLC** and **capillary zone electrophoresis (CZE)** procedures to analyze some non-aromatic organic acids in floral honeys.

- **Precision**
- **Recovery**
- **Specificity**
- **Sensitivity**
- **Simplicity**
- **Speed**
- **Cost**

GEOGRAPHICAL ORIGIN OF THE SAMPLES

50 Samples of honey





NW SPAIN

GALICIA

BOTANICAL ORIGIN OF THE SAMPLES

Melissopalinology

Honey sediment:

glycerined methyl-green hydroalcoholic solution

Terradillos et al. 1994. Bee Science 3

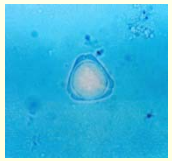
Identification, counting and presentation of frequencies

Louveaux et al. 1978. Bee World 59

Von der Ohe et al. 2004. Apidologie 35

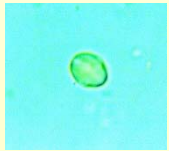
frequency classes

25 multifloral honeys

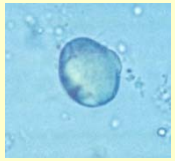


21 eucalyptus honeys (*Eucalyptus* sp.)

3 chestnut honeys (*Castanea sativa*)



1 clover honey (*Trifolium* sp.)



ENZYMATIC METHODS

ENZYMATIC METHODS

Measurements at 340 nm

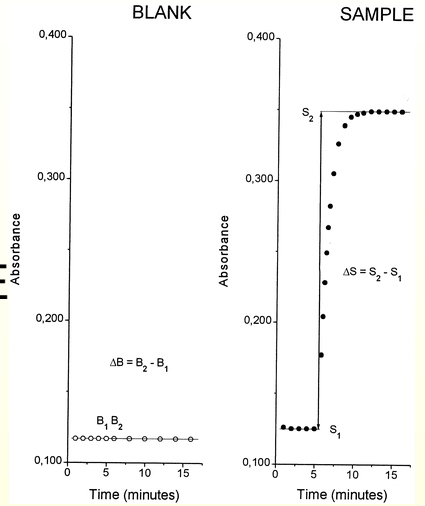
Total D-Gluconic acid

(Mato et al. 1997. J. Agric. Food Chem. 45)

Gluconate kinase

6-phosphogluconate dehydrogenase

NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE



Citric acid *(Mato et al.1998. J. Agric. Food Chem. 46)*

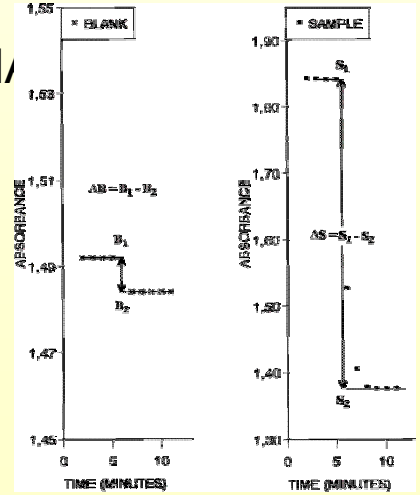
Citrate liase

L-malate dehydrogenase

L-lactate dehydrogenase

REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE (NA

Previous clarification with polyvinylpolypyrrolidone (PVPP)



L-Malic acid *(Mato et al. 1998. Food Chem. 62)*

Glutamate-oxaloacetate transaminase

L-malate dehydrogenase

NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD)

ENZYMATIC METHODS

Total D-Gluconic acid

Precision

	HONEY SAMPLE		
	A	B	C
	3.90	7.28	11.72
	3.92	7.28	11.71
	3.92	7.25	11.70
	3.92	7.25	11.71
	3.90	7.28	11.72
	3.92	7.26	11.72
	3.92	7.29	11.71
	3.90	7.28	11.70
	3.90	7.28	11.72
	3.93	7.25	11.70
mean	3.91	7.27	11.71
SD[a]	*0.0116*	*0.0156*	*0.00876*
% CV[b]	*0.30*	*0.22*	*0.07*

[a] Standard deviation. [b] Coefficient of variation.

Recovery

present	added (g/kg)	found (g/kg)	recovery (%)
	2.00	3.96	100.0
	2.00	3.97	100.5
	2.00	3.96	100.0
	4.00	6.00	100.3
	4.00	5.99	100.0
	4.00	5.94	99.8
1.96			
	8.00	9.94	99.8
	8.00	9.91	99.4
	8.00	9.90	99.3
	10.00	11.90	99.4
	10.00	11.88	99.2
	10.00	11.94	99.8
mean			99.8
SD[a]			*0.403*
% CV[b]			*0.40*

[a] Standard deviation. [b] Coefficient of variation.

RESULTS (g/kg)

Mean: 7.37
Standard deviation: 2.92
Spread of values: from 2.38 to 13.53

ENZYMATIC METHODS

Citric acid

Precision: Study of precision of the determination of **citric acid (mg/kg)**, using the direct enzymatic method and the enzymatic method with previous clarification with PVPP

	Honey samples					
	A		B		C	
	direct	clarified	direct	clarified	direct	clarified
n	*10*	*10*	*10*	*10*	*10*	*10*
mean	44.9	44.2	424.0	428.4	817.2	827.0
SD[a]	*1.19*	*0.707*	*6.497*	*2.198*	*8.323*	*2.186*
% CV[b]	*2.66*	*1.60*	*1.53*	*0.51*	*1.02*	*0.26*

[a] *Standard deviation.* [b] *Coefficient of variation.*

Recovery: Study of the recovery of the determination of **citric acid (mg/kg)**, using the direct enzymatic method and the enzymatic method with previous clarification with PVPP

Sample A	Recovery (%)	
added (mg/kg)	direct	clarified
25	104.0	99.6
175	100.7	100.4
375	99.8	100.9
775	97.3	100.9
mean	100.5	100.5
SD[a]	*2.769*	*0.614*
% CV[b]	*2.76*	*0.61*

[a] *Standard deviation.* [b] *Coefficient of variation.*

RESULTS (mg/kg)

Mean: 116.3
Standard deviation: 116.0
Spread of values: from 20.7 to 451.2

ENZYMATIC METHODS

Malic acid

Precision: Study of precision of the determination of **malic acid (mg/kg)**

	Honey samples			
	A	B	C	D
n	*10*	*10*	*10*	*10*
mean	94	240	463	596
SD[a]	*3.31*	*5.39*	*5.17*	*4.60*
% CV[b]	***3.5***	***2.2***	***1.1***	***0.8***

[a]Standard deviation. [b]Coefficient of variation.

RESULTS (mg/kg)

Mean: 91.0
Standard deviation: 132.0
Spread of values: from 8.0 to 578.0

Recovery: **Malic acid (mg/kg)**

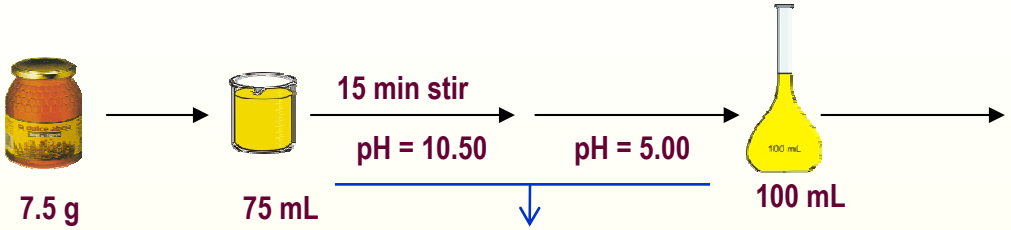
present (mg/kg)	added (mg/kg)	found (mg/kg)	recovery (%)
	50	99	104
	50	95	96
	50	99	104
	250	288	96
	250	300	101
	250	305	103
47			
	450	480	96
	450	511	103
	450	485	97
	650	681	98
	650	669	96
	650	713	102
n			12
Mean			100
SD[a]			*3.45*
% CV[b]			***3.5***

[a] Standard deviation. [b] Coefficient of variation.

HPLC PROCEDURE

(Suárez-Luque et al. 2002. J. Chrom A 955)

HPLC PROCEDURE



10 ml

↓

Filtration through 0.45 um cellulose acetate membrane

↓

SPE

20 ul | Triplicate

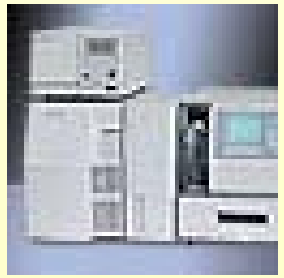
SOLID-PHASE EXTRACTION (SPE)
ANION-EXCHANGE CARTRIDGE

Activation: 10 ml NaOH 0.1 *M*
percolation rate: 3 ml/min

Sample: **10 ml**
flow-rate: 0.5 ml/min

Cartridge washing: 10 ml water (3 ml/min)

Elution of organic acids: 4 ml H_2SO_4 0.1 M (0.5 ml/min)



HPLC PROCEDURE

CHROMATOGRAPHIC CONDITIONS

Column: Spherisorb ODS-2 S5
Temperature: 25 ^{o}C
Mobile phase: Metaphosphoric acid (pH 2.20)
Flow-rate: 0.7 ml/min
Detection: 215 nm
Time of analysis: **15 minutes**

ORGANIC ACIDS DETERMINED

Malic
Maleic
Citric
Succinic
Fumaric

HPLC PROCEDURE

Retention times

Organic acid	Retention time (min)±SD
Malic	5.81 ± 0.01
Maleic	9.07 ± 0.10
Citric	10.87 ± 0.09
Succinic	11.81 ± 0.10
Fumaric	13.61 ± 0.10

Parameters and correlation coefficients (*r*) of calibration plots (y= *ax* + *b*)

y= peak height *x= amount of acid (mg/kg)*

Organic acid	*a*	*b*	*r*
Malic	19.79	45.79	1.0000
Maleic	1272	41.66	1.0000
Citric	19.65	0.5058	0.9999
Succinic	6.984	46.70	0.9994
Fumaric	1401	38.05	0.9998

Detection and quantification limits

Organic acid	Detection limit (mg/kg)	Quantification limit (mg/kg)
Malic	1.55	2.93
Maleic	0.059	0.075
Citric	1.44	2.72
Succinic	7.57	10.93
Fumaric	0.0064	0.025

HPLC PROCEDURE

Precision

Repeatability

Acid	Repeatability (n=5)					
	Sample 1		Sample 2		Sample 3	
	Mean±SD (mg/kg)	RSD (%)	Mean±SD (mg/kg)	RSD (%)	Mean±SD (mg/kg)	RSD (%)
Malic	35,5±1,9	2,29	109,4±2,9	2,67	274,1±8,8	3,20
Maleic	0.213±0.011	0.93	0.257±0.008	3.11	0.143±0.003	1.93
Citric	70,9±1,6	0,90	120,8±0,8	0,70	390,3±10,5	2,68
Succinic	23,44±0,04	0,27	31,2±0,22	0,71	152,9±3,1	2,02
Fumaric	0.130±0.008	1.21	1.011±0.007	0.68	6.88±0.24	2,94

less than

Reproducibility

Acid	Reproducibility (n=3)					
	Sample 1		Sample 2		Sample 3	
	Mean±SD (mg/kg)	RSD (%)	Mean±SD (mg/kg)	RSD (%)	Mean±SD (mg/kg)	RSD (%)
Malic	34,6±1,0	2.95	113,1±3,4	3,03	268,0±10,4	3,90
Maleic	0.203±0.008	3.72	0.252±0.011	4.40	0.147±0.004	2.57
Citric	69,5±3,2	4,68	122,3±3,2	2,59	378,4±12,0	3,18
Succinic	23,10±1,12	4,86	30,69±0,92	2,99	149,4±3,4	3,07
Fumaric	0.128±0.005	4.15	0.987±0.045	4.59	7.08±0.09	3,23

HPLC PROCEDURE

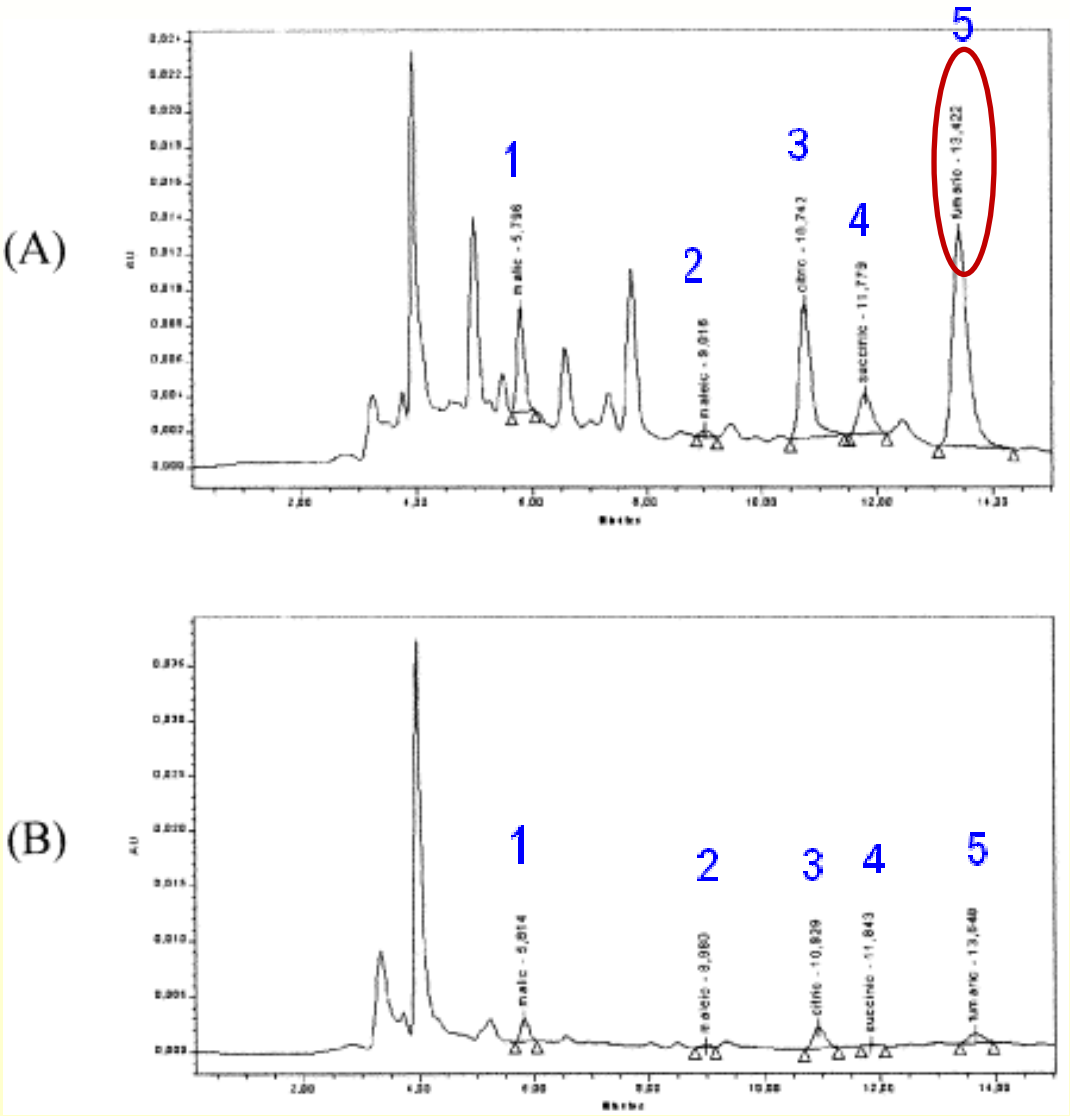
Standard solutions recoveries after solid-phase extraction procedure

Organic acid	Recovery (%) (mean±SD)	RSD (%)
Malic	101,8±0,18	0,18
Maleic	103,3±0,099	0,10
Citric	100,8±0,085	0,08
Succinic	99,2±0,34	0,34
Fumaric	103,4±1,43	1,38

Recovery of **carboxylic acids** added to honey after solid-phase extraction procedure

Organic acid	Recovery (%) (mean±SD)	RSD (%)
Malic	62,9±4,4	7,0
Maleic	93,4±8,2	8,8
Citric	99,4±1,5	1,5
Succinic	75,0±5,0	6,7
Fumaric	94,4±4,6	4,9

HPLC PROCEDURE



(A) *Castanea sativa* honey

1 Malic acid
2 Maleic acid
3 Citric acid
4 Succinic acid
5 Fumaric acid

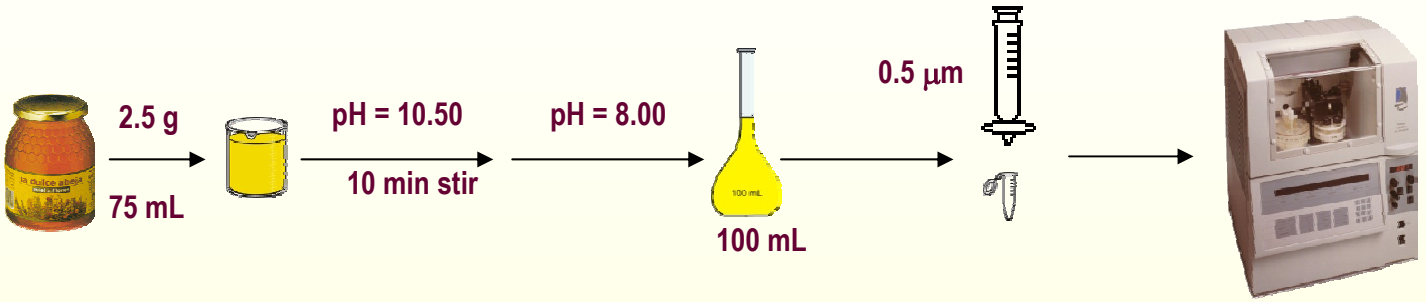
(B) Multifloral honey

CAPILLARY ZONE ELECTROPHORESIS (CZE)

(Mato et al. 2006. J. Agric. Food Chem. 54)

CAPILLARY ZONE ELECTROPHORESIS

TREATMENT OF THE HONEY SAMPLE



ELECTROPHORETIC CONDITIONS

Sample injection (in TRIPLICATE)	Hydrodynamic mode (10 cm elevation) Time of injection: 30 seconds Quantity of sample: 37.2 nL
Electrolyte composition	7.5 m*M* NaH_2PO_4; 2.5 m*M* Na_2HPO_4; 2.5 m*M* TTAOH; 0.24 m*M* $CaCl_2$ (pH = 6.40)
Separation	Capillary column: 60 cm x 75 μm ID Temperature: 25 ºC Running voltage: -25 kV
Detection	UV DIRECT (185 nm)
Time of analysis	4 minutes

CAPILLARY ZONE ELECTROPHORESIS

ORGANIC ACIDS DETERMINED

Oxalic
Formic
Malic
Succinic
Pyruvic
Acetic
Lactic
Citric
Gluconic

CAPILLARY ELECTROPHORESIS

	LOD (mg/kg)	LOQ (mg/kg)	CALIBRATION PLOTS [y=ax+b (r)]	
OXALIC ACID	0.4	12	y = 26.7x + 502	(0.9999)
FORMIC ACID	2.1	23	y = 10.7x + 212	(0.9996)
MALIC ACID	2.0	21	y = 10.8x + 201	(0.9997)
SUCCINIC ACID	2.0	12	y = 13.4x + 294	(0.9998)
PYRUVIC ACID	7.0	39	y = 9.8x + 335	(0.9996)
ACETIC ACID	11	34	y = 17.9x + 233	(0.9999)
LACTIC ACID	4.2	26	y = 9.6x + 204	(0.9999)
CITRIC ACID	9.2	28	y = 12.8x - 533	(0.9996)
GLUCONIC ACID	38	78	y = 5.3x + 56	(0.9999)

LOD= detection limit
LOQ= quantification limit
y= peak area
x= amount of organic acid (mg/kg)
Calibration test: In triplicate

CAPILLARY ZONE ELECTROPHORESIS

Precision

Repeatability

Injection of the honey sample 5 times

Relative standard deviations (RSDs):

0.2% (lactic acid) - 4.6% (formic acid)

Reproducibility

Analysis of each honey sample on 3 different days over 1 month.

Relative standard deviations (RSDs):

0.5% (acetic acid) – 10.0% (oxalic acid)

Recovery

2 honey samples: Low organic acid contents.

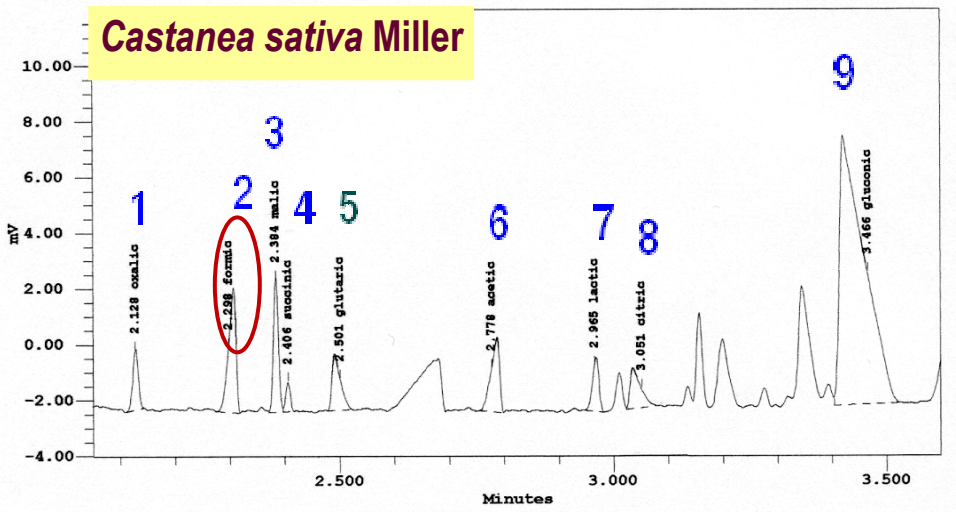
Adding three increasing amounts of an organic acid standard mixture to a half amount of honey (1.25 g)

Recoveries (mean (%) ± SD)

89.4 ± 10.1 (citric acid) – 104.6 ± 4.8 (acetic acid)

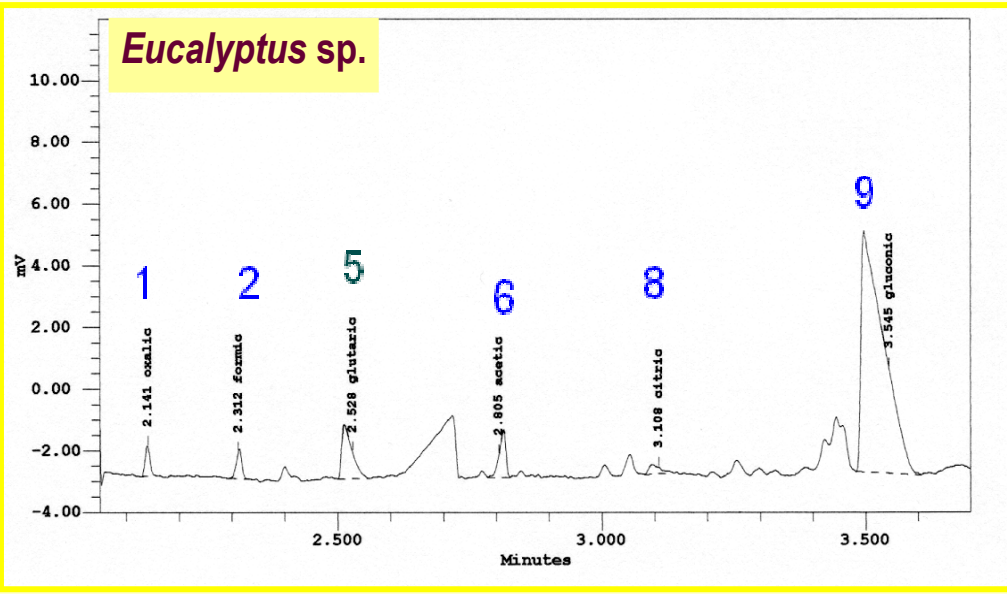
CAPILLARY ZONE ELECTROPHORESIS

***Castanea sativa* Miller**



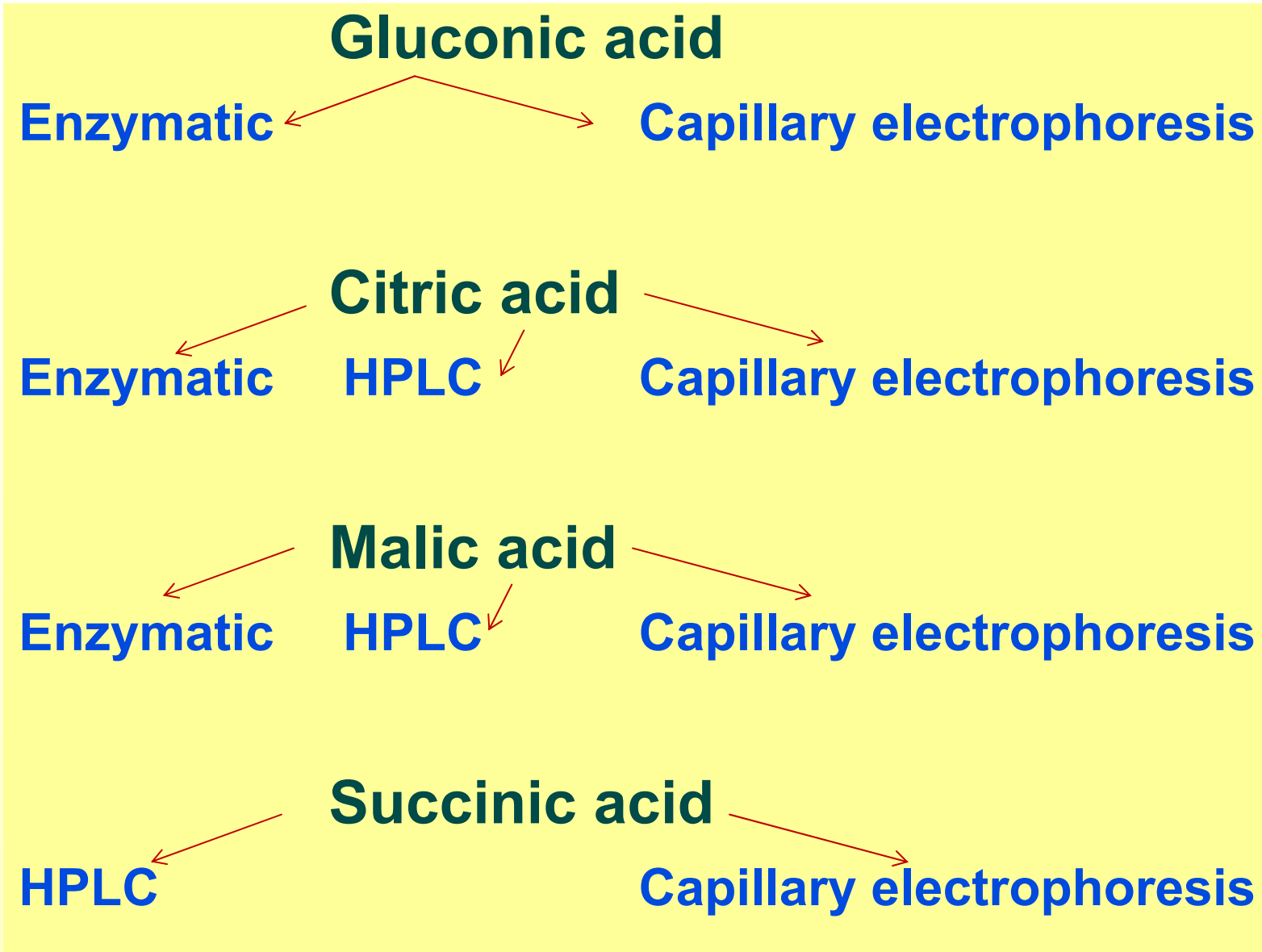
1 Oxalic acid
2 Formic acid
3 Malic acid
4 Succinic acid
5 Glutaric acid (reference acid to calculate the relative migration times)
6 Acetic acid
7 Lactic acid
8 Citric acid
9 Gluconic acid

***Eucalyptus* sp.**



COMPARISON OF METHODS

NON-AROMATIC ORGANIC ACIDS



STATISTICAL ANALYSIS

SPSS for Windows v. 10.0.6 SPSS Inc. (1999)

Purposes:

1.- To estimate if the methods (enzymatic, HPLC and CZE) lead to the same results.

Correlation among the results obtained by the three methods.

Comparison of values: *t-test* at a confidence level of 99%.

2.- To propose a method of choice.

GLUCONIC ACID (g/kg)

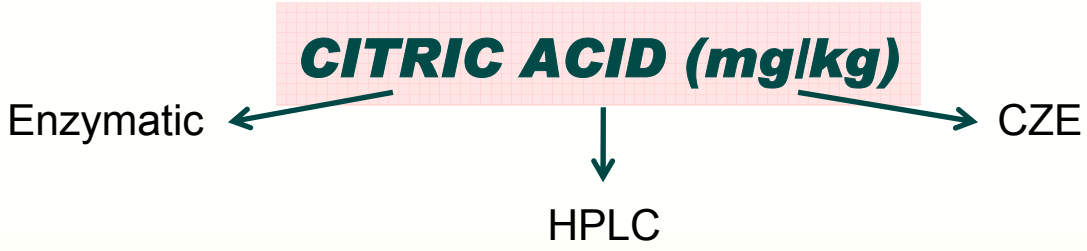
Enzymatic CZE

1) There is a **significant correlation** between the values of gluconic acid between both methods (*r*= 0.998).

2) Student's t test showed that **there are not significant differences** ($p > 0.01$) between the results obtained by both the enzymatic method and the CZE procedure.

Enzymatic method: HIGHER { specificity; precision and accuracy; sensitivity }

CZE method: Numerous organic acids are determined simultaneously

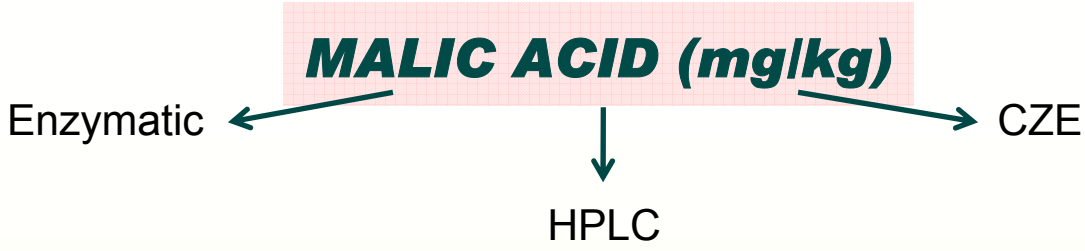


1) There is a **significant correlation** among the values of citric acid obtained by the three methods (the lowest *r* was 0.990).
The **best correlation** was obtained between enzymatic and HPLC mehtods (*r*= 0.993).

2) Student's t test showed that **there are not significant differences** ($p > 0.01$) between:
- All the results obtained by both enzymatic and HPLC methods.
- All the results obtained by HPLC and CZE methods.
- The results obtained by both enzymatic and CZE methods, but only for concentrations lower than 250 mg/kg.

Enzymatic method: HIGHER
- specificity
- simplicity
- precision

HPLC method:
- HIGHER sensitivity
- Other organic acids are determined simultaneously.



1) There is a **significant correlation** among the values of malic acid obtained by the three methods (the lowest *r* was 0.947).
The **best correlation** was obtained between enzymatic and CZE mehtods (*r*= 0.999).

2) Student's t test showed that **there are not significant differences** (p > 0.01) between the results obtained by the three methods.

Enzymatic method: HIGHER { specificiy; sensitivity

CZE method: Numerous organic acids are determined simultaneously

Better { *precision*; *accuracy*

SUCCINIC ACID (mg/kg)

HPLC CZE

There is **no significant correlation** between the values of succinic acid between both methods.

Values of succinic acid are considerably higher by HPLC

Interference

CZE method:

- No interferences
- Excellent recoveries

CONCLUSIONS

Gluconic acid has been determined by enzymatic and CZE methods. Both methods lead to the same results at a confidence level of 99% for the range of values studied. Enzymatic method provides **greater specificity and sensitivity**, whereas CZE has the advantage of **determining a profile of non-aromatic organic acids**. Precision and accuracy have been similar with both methods, but slightly better with the enzymatic procedure.

Citric acid has been determined by enzymatic, HPLC, and CZE methods. All methods are comparable, at a confidence level of 99%, for concentrations lower than 250 mg/kg. For any concentration, the **methods of choice** would be enzymatic and HPLC. Enzymatic method provides **greater specificity**, **precision** and **simplicity**. HPLC procedure gives **higher sensitivity** and **a profile of other minority non-aromatic organic acids**.

Malic acid has been determined by enzymatic, HPLC, and CZE methods. All methods lead to the same results at a confidence level of 99% for the range of values studied. Enzymatic method provides **greater specificity and sensitivity**, whereas CZE has the advantage of **determining a profile of non-aromatic organic acids**. Precision and accuracy have been similar with both methods, but slightly better with the CZE procedure.

Succinic acid has been determined by HPLC, and CZE methods, which are not comparable. The **method of choice** would be CZE, because **there are not interferences** and the procedure provides **excellent recoveries**.

MANY THANKS FOR YOUR ATTENTION

M. Teresa Sancho
University of Burgos
Food Science Department
Plaza Misael Bañuelos s/n
09001 Burgos (SPAIN), EU
Phone: + 34-947-258-813
FAX: + 34-947-258-831
E-mail: mtsancho@ubu.es